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# Ethanol and Isopropanol in Concentrations Present in Hand Sanitizers Sharply Reduce Excystation of *Giardia* and *Entamoeba* and Eliminate Oral Infectivity of *Giardia* Cysts in Gerbils

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**Enteric protozoan parasites, which are spread by the fecal-oral route, are important causes of diarrhea (*Giardia duodenalis*) and amebic dysentery (*Entamoeba histolytica*). Cyst walls of *Giardia* and *Entamoeba* have a single layer composed of fibrils of β-1,3-linked GalNAc and β-1,4-linked GlcNAc (chitin), respectively. The goal here was to determine whether hand sanitizers that contain ethanol or isopropanol as the active microbicide might reduce transmission of these parasites. We found that treatment with these alcohols with or without drying in a rotary evaporator (to model rapid evaporation of sanitizers on hands) kills 85 to 100% of cysts of *G. duodenalis* and 90 to 100% of cysts of *Entamoeba invadens* (a nonpathogenic model for *E. histolytica*), as shown by nuclear labeling with propidium iodide and failure to excyst *in vitro*. Alcohols with or without drying collapsed the cyst walls of *Giardia* but did not collapse the cyst walls of *Entamoeba*. To validate the *in vitro* results, we showed that treatment with alcohols eliminated oral infection of gerbils by 1,000 *G. duodenalis* cysts, while a commercial hand sanitizer (Purell) killed *E. invadens* cysts that were directly applied to the hands. These results suggest that expanded use of alcohol-based hand sanitizers might reduce the transmission of *Giardia* and *Entamoeba*.**

*Giardia duodenalis* (also known as *Giardia lamblia*) is an important cause of human diarrhea in both the developing and developed world and is an important zoonosis of companion and production animals (1–8). *Entamoeba histolytica* may cause amebic dysentery and liver abscess, primarily in the developing world (9–11). Because these parasites may cause severe infections and are readily transmitted by asymptomatic carriers, the National Institute of Allergy and Infectious Diseases (NIAID) has designated *Giardia* and *Entamoeba* category B priority pathogens.

Our laboratory has extensively characterized the cyst walls of *Giardia* and *Entamoeba*, each of which contains a prominent sugar polymer and a small set of proteins (12, 13). In contrast, the *Saccharomyces cerevisiae* cell wall contains two sugar polymers (chitin and β-1,3-linked glucan) and ~100 glycoproteins (14). The *Giardia* cyst wall contains curled fibrils of β-1,3-linked *N*-acetylgalactosamine (GalNAc) and cyst wall proteins (CWPs) that bind the GalNAc homopolymer (15, 16). Cyst wall proteins have very short Asn-linked glycans composed of two GlcNAc residues (17). While cytosolic proteins of *Giardia* may contain GlcNAc linked to Ser or Thr residues, no *O*-linked glycans have been identified in its secreted or cyst wall proteins (18).

The *Entamoeba* cyst wall is modeled by *Entamoeba invadens*, which infects reptiles but not humans, because *E. histolytica* does not encyst in axenic culture or in the mouse model. The *Entamoeba* cyst wall is composed of fibrils of β-1,4-linked *N*-acetylglucosamine (GlcNAc) (chitin), which are labeled by wheat germ agglutinin (WGA) (19, 20). *Entamoeba* cyst walls contain three abundant chitin-binding glycoproteins (lectins) that cross-link chitin, degrade chitin, or self-aggregate (21). These glycoproteins contain *N*-glycans that are longer than those of *Giardia* but shorter than those of the host. Cyst wall glycoproteins also contain unique *O*-phosphodiester-linked glycans (22). When cysts of *Giardia* or *Entamoeba* are placed in excystation media that mirror conditions in the small intestines, trophozoites (the motile, colonizing form) are released (23, 24).

We are interested in alcohol-based hand sanitation methods to reduce transmission of *Giardia* and *Entamoeba*, because these sanitizers, which were identified >100 years ago, are effective against many viral, bacterial, and fungal infections; alcohol-based sanitizers are relatively inexpensive and do not depend upon scarce supplies of clean water; and there are no human vaccines for *Giardia* and *Entamoeba* (25–32). Hand sanitizers contain 63% to 80% ethanol or isopropanol. They kill microorganisms by penetrating walls, disrupting membranes, and denaturing proteins. Here, we show that alcohol treatment blocks excystation of *G. duodenalis* and *E. invadens in vitro* and blocks oral infection of gerbils by *G. duodenalis* cysts.

## MATERIALS AND METHODS

***Giardia duodenalis.*** Cysts of the H3 strain of *G. duodenalis* (assemblage B), which are produced in gerbils, were purchased from Waterborne, Inc. (New Orleans, LA). The cysts (1,000 cysts/200 μl) were incubated with occasional shaking in water, 63% ethanol, 80% ethanol, 63% isopropanol, or 80% isopropanol for 5 min at room temperature (~21°C) with or

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without drying in a rotary evaporator (Savant SpeedVac SC110A Plus concentrator; Thermo Fisher Scientific, Waltham, MA) for 5 min. These times, which are longer than those used to wash hands with alcohol-based hand sanitizers, allowed us to process multiple samples as a group and so avoid the variability that might occur when samples were processed sequentially. WHO formulations for hand sanitizers are available online (http://www.who.int/gpsc/5may/Guide_to_Local_Production.pdf). Cyst walls were labeled for 1 h at room temperature with an anti-CWP1 monoclonal antibody (MAb) (Waterborne Inc., New Orleans, LA), which was diluted 1:500 in phosphate-buffered saline (PBS) (16). After washing three times in PBS, the cysts were incubated with 4′,6-diamidino-2-phenylindole (DAPI) (0.2 μg/ml) and/or propidium iodide (PI) (0.2 μg/ml) for 5 min at room temperature and then washed twice in PBS. DAPI is a vital stain that penetrates membranes and binds nuclei, although it may be excluded by cyst walls. PI, which is often included in LIVE/DEAD kits, cannot penetrate intact membranes and so labels only nuclei of dead cells. Cysts and trophozoites were visualized with a DeltaVision deconvoluting microscope (Applied Precision, Issaquah, WA). Images were taken with a 100× objective and deconvolved using Applied Precision's softWoRx software (16).

*G. duodenalis* excystation was induced by incubating cysts in excystation medium containing 1 mg/ml chymotrypsin in Tyrode salt solution for 30 min at 37°C (23). Excystation rates were determined by counting intact versus excysted walls with a Labophot phase-contrast microscope (Nikon Instruments, Inc., Melville, NY) using a 20× objective. In each experiment, 100-μl aliquots that contained ~100 cysts were counted for each sample, and the experiments were performed at least 3 times each. The rate of excystation for each alcohol treatment was compared with that of cysts incubated in water using an unpaired *t* test (GraphPad Prism software). Similarly, the rate of excystation for each alcohol treatment plus drying with the rotary evaporator was compared with that of cysts incubated in water and then dried. No statistical comparisons were made between groups treated with different alcohols. Excysted *Giardia* trophozoites were cultured in TYI-S-33 medium at 37°C to confirm their viability (33).

Four- to 6-week-old female gerbils (Charles River Laboratories, Wilmington, MA), none of which were infected with *Giardia*, were experimentally infected by oral gavage with 1,000 *G. duodenalis* strain H3 cysts in 100 μl water (34). The cysts were untreated, incubated in 63% or 80% ethanol and dried, or incubated in 80% isopropanol and dried. After 7 days of infection, the gerbils were euthanized by $CO_2$ asphyxiation, and the duodenum was removed, minced, and incubated in 1 ml TYI-S-33 medium for 60 min to release *Giardia* trophozoites, which were counted with phase microscopy and a hemocytometer. Three aliquots from each animal were counted with a hemocytometer. Four animals were in each experimental group, and the experiments were performed two times each. The averages and standard deviations for 8 animals in each group are presented in Fig. 1I. No statistical test was used, as we were unable to identify any *Giardia* organisms in the lumen of the duodenum in gerbils infected with cysts treated with ethanols or isopropanol.

The manipulation of *G. duodenalis* cysts was approved by the Boston University Institutional Biosafety Committee. Infecting gerbils with *G. duodenalis* cysts was approved by the Boston University Institutional Animal Care and Use Committee.

***Entamoeba invadens.*** The IP-2 strain of *E. invadens* was cultured in TYI-SS medium at room temperature, and trophozoites ($10^5$/ml) were induced to encyst by transfer to medium with reduced glucose, osmolarity, and serum for 72 h (20, 33, 35). After encystation, the cysts were suspended in sterile water and left at 4°C for 6 h to lyse trophozoites. *E. invadens* cysts were treated with alcohols, and then DAPI and PI labeling of the cysts were performed as described for *Giardia*, except cyst walls were labeled with 10 μg/ml WGA, which binds to chitin fibrils (21). *E. invadens* excystation rates were determined by incubating $10^4$ cysts in 10 ml of excystation medium containing 40 mM sodium bicarbonate and 1.25 mM taurodeoxycholate for 24 h at room temperature and counting intact versus excysted walls with the phase microscope (24). Alternatively, ~$10^7$ *E. invadens* cysts in 1 ml of water were applied with a pipette to the index and pointer fingers of one hand, and hands were washed to dryness (~2 min) with 1 to 2 ml of a commercial hand sanitizer (Purell; Gojo Industries, Akron, OH). Purell contains 70% ethanol. Residual cysts were eluted with 300 ml of water; concentrated to 1 ml by centrifugation; and labeled with PI, DAPI, and WGA, as described above. The numbers of live cysts (with DAPI staining but no PI staining) and dead cysts (with PI-stained nuclei or walls torn apart) recovered from the hands were compared to those of untreated *E. invadens* cysts. Hands were extensively washed with soap and water, and all wash solutions were decontaminated with Wescodyne germicidal detergent (Steris, Mentor, OH).

## RESULTS

**Ethanol and isopropanol permeabilize membranes of *G. duodenalis* trophozoites within cysts, collapse cyst walls, dramatically reduce excystation *in vitro*, and block oral infection of gerbils.** The protocol for testing the effects of alcohols on the infectivity of *Giardia* cysts is shown in Fig. 1A. All *G. duodenalis* cysts, regardless of experimental treatment, labeled with a MAb to the most abundant cyst wall protein (CWP1) (Fig. 1B). In contrast, only ~20% of *G. duodenalis* cysts, which were washed in PBS and incubated in water, labeled with DAPI, a nuclear stain that penetrates membranes. This result shows that the intact cyst wall is relatively impermeable to small molecules, as the formula weight of DAPI is ~227 and DAPI readily penetrates intact plasma membranes of live trophozoites (data not shown). Zero to 5% of these cysts labeled with PI (the "DEAD" stain in LIVE/DEAD kits), depending upon the batch of commercially obtained cysts (Fig. 1B). Cysts that had been incubated in water remained viable (they might label with DAPI but not with PI) after being quickly dried with the rotary evaporator (Fig. 1C). In contrast, drying overnight in the cold decreased the viability of *G. duodenum* cysts (36).

After treatment for 5 min with ethanol or isopropanol, nuclei of >95% of *G. duodenalis* trophozoites within cysts label with PI, indicating that the trophozoites within are dead (Fig. 1D to G). Staining with the anti-CWP1 MAb shows that cyst walls are often collapsed by incubation with ethanols. Drying in the rotary evaporator modestly improved killing. Supporting the idea that alcohols kill *G. duodenalis* cysts, ethanol or isopropanol treatment with or without drying dramatically reduces excystation *in vitro* to 0 to 15% of parasites examined (Fig. 1H). The effect of each alcohol treatment on excystation was highly significant ($P < 0.001$) compared with treatment in water. No statistical comparison was made between the alcohol treatments, as they all worked well to block excystation. Excysted parasites are viable, as shown by their motility and by their growth in culture. Consistent with the absence of PI labeling of their nuclei (Fig. 1C), drying of water-treated cysts in the rotary evaporator did not prevent excystation.

Treatment of 1,000 *Giardia* cysts with 63% and 80% ethanol or 80% isopropanol, all with drying in the rotary evaporator, completely prevents gerbil infection by oral gavage, which was determined by counting trophozoites in the duodenal lumen after 1 week of infection (Fig. 1I) (34). Eight of 8 gerbils were infected with untreated cysts, and we recovered >$10^7$ *G. duodenalis* trophozoites (average) from their duodenums. In contrast, we were unable to recover any trophozoites from 24 of 24 gerbils infected with alcohol-treated cysts (the lowest detection limit with the hemocytometer was $10^4$ parasites/ml).

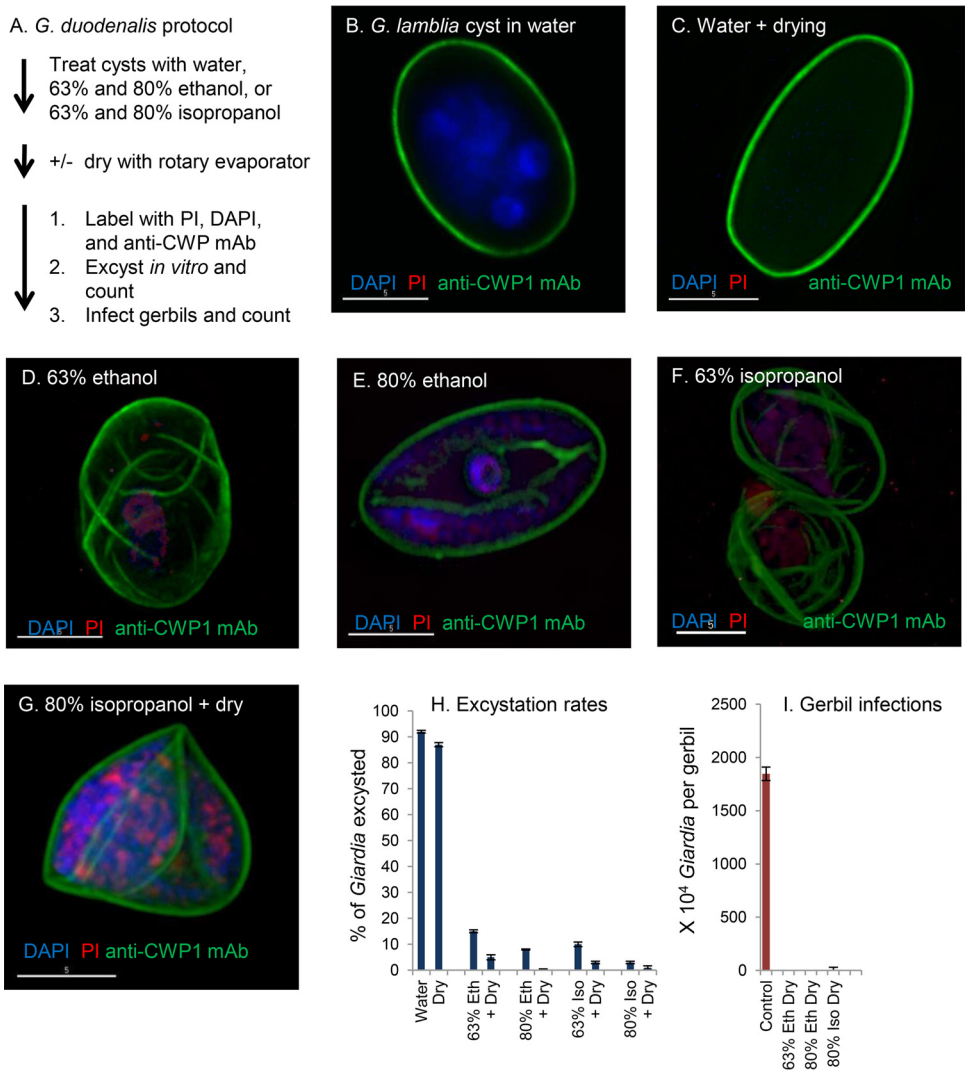


**FIG 1** Ethanol and isopropanol collapse cyst walls of *G. duodenalis*, permeabilize membranes of trophozoites within cysts, dramatically reduce excystation *in vitro*, and block oral infection of gerbils. (A) Protocols for testing the effects of ethanol on cysts of *G. duodenalis*. (B and C) The vital stain DAPI (blue), but not PI ("DEAD" stain) (red), labels nuclei of some cysts (B), but not others (C), in water. Note that drying after incubation in water has no effect on viability. Cyst walls were labeled green with an anti-CWP1 monoclonal antibody. (D to G) After treatment with 63% ethanol (D), 80% ethanol (E), 63% isopropanol (F), or 80% isopropanol plus drying (G), nuclei of *G. duodenalis* cysts were labeled with PI and walls appeared to collapse. (H and I) Alcohol treatments reduced excystation (H) and blocked oral infection of gerbils with *G. duodenalis* cysts (I). (H) Excystation plot showing averages and standard errors from at least 3 experiments with hundreds of cysts counted per experiment. In each case, the *P* values were $<0.001$ when excystation rates for ethanol (Eth) treatments versus water or for ethanol treatments plus drying versus water plus drying were compared. Iso, isopropanol. (I) Gerbil infections showing averages and standard errors from eight gerbils per treatment (performed in two separate experiments). Because we were unable to recover any *Giardia* organisms from gerbils infected with alcohol-treated cysts, no statistical analysis was performed.

**Alcohols in the test tube and on the hands kill *E. invadens* cysts, as shown by PI staining, disruption of cyst walls, and/or reduced rates of excystation.** The protocols for testing the effects of alcohols on the infectivity of *Entamoeba* cysts are shown in Fig. 2A and G. All the control *E. invadens* cysts in water labeled with WGA (chitin in the wall) and DAPI but did not label with PI (Fig. 2B). Treatment of *E. invadens* cysts with ethanol or isopropanol makes the cysts permeable to PI (Fig. 2C to E). While dehydration with alcohols collapses *G. duodenalis* cyst walls (Fig. 1D to G), alcohol treatment and drying do not collapse the cyst wall of *E. invadens*. Alcohol treatment with or without drying with the rotary evaporator blocks excystation *in vitro* of *E. invadens* cysts (Fig. 2F). Again, the difference between the excystation rates of alcohol-treated organisms and that of cysts incubated in water was highly significant ($P < 0.001$). As described for *G. lamblia* cysts, drying of water-treated *E. invadens* cysts in the rotary evaporator did not prevent excystation.

When *E. invadens* cysts, which are not infectious to people, are applied to the index and pointer fingers and the hands are cleaned with a commercial hand sanitizer (Purell), the cysts are killed, as shown by PI staining of nuclei (Fig. 2H). In addition, cyst walls, which are heavily contaminated with squamous epithelial cells and bacteria, are frequently disrupted (Fig. 2I). Because of this heavy contamination, we did not perform excystation experiments but instead counted >500 Purell-treated cysts (total) in three separate experiments. All of these cysts appeared dead (they were labeled with PI

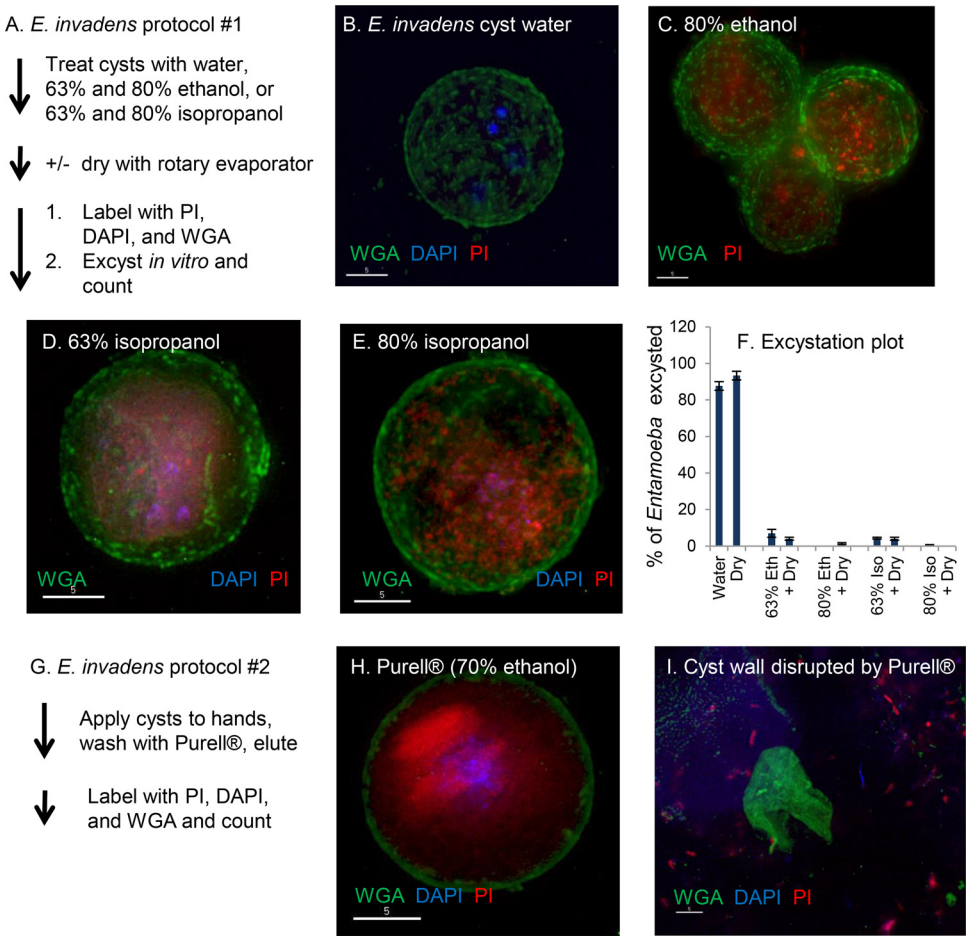


**FIG 2** Alcohols in the test tube and on the hands kill *E. invadens* cysts, as shown by PI staining, reduced rates of excystation, and/or disruption of cyst walls. (A) Protocol for testing the effects of ethanol-based hand sanitizers on *E. invadens* cysts in the test tube. (B) DAPI (blue), but not PI (red), labels nuclei of control cysts in water. (C to E) Cyst walls were stained green with WGA, which binds to chitin fibrils. After treatment with 80% ethanol (C), 63% isopropanol (D), or 80% isopropanol (E), nuclei of *E. invadens* cysts were labeled with PI, indicating the trophozoites within were dead. (E) Drying with the rotary evaporator caused some *E. invadens* cysts to form large aggregates. (F) Treatment of cysts in the test tube with ethanol or isopropanol with or without drying with the rotary evaporator dramatically reduced excystation *in vitro*. (G) Protocol for testing the effect of a commercial hand sanitizer (Purell) on *E. invadens* applied directly to the hands. (H and I) Purell killed *E. invadens* cysts that were directly applied to the hands, as shown by PI labeling (H) or by disruption of the cyst walls (I). Heavy contamination with squamous epithelial cells and bacteria made it impossible to judge excystation, so >500 PI-, DAPI-, and WGA-labeled cysts were examined in three separate experiments with Purell. In no case were we able to identify any intact cysts, as defined by DAPI and WGA labeling and failure to label with PI (as in panel B).

and/or had disrupted walls), and none appeared alive (i.e., labeled with DAPI but not PI and had intact walls).

## DISCUSSION

**Arguments for using alcohol-based hand sanitizers to reduce the spread of *Giardia* and *Entamoeba*.** As shown by the labeling of the nuclei of alcohol-treated cysts with PI, alcohols penetrate cyst walls and disrupt plasma membranes of trophozoites of *G. duodenalis* and *E. invadens* that are inside (Fig. 1 and 2). Alcohols dramatically reduce excystation of both parasites and block gerbil infections with 1,000 *G. duodenalis* cysts, which is well above the minimal infectious dose (34). As the vast majority of *Giardia* and *Entamoeba* organisms were killed by alcohols without drying, the effect of the rotary evaporator was minimal. The effect of alcohol treatment on *G. duodenalis* infections is much greater than that of oral consumption of whole wheat and/or wheat germ, which contain WGA and are common components of the human diet. In a rodent model and in human infections, WGA, which binds to short *N*-glycans of *G. duodenalis* and reduces excystation *in vitro*, showed modest reduction in cysts shed and in symptoms (humans) (37–39). A commercial alcohol-based hand sanitizer killed *E. invadens* cysts directly applied to the hands, validating the findings when *E. invadens* cysts were treated with alcohols in a microcentrifuge tube and dried in the rotary evaporator.

The alcohol-based hand sanitizers are safe, relatively inexpensive, and well tolerated; they do not need water; they do not select for antibiotic-resistant organisms; they also reduce infections with viruses, bacteria, and fungi; and they are packaged in small volumes that are stable at room temperature and so can be used by travelers (25–32, 40). While hand washing with soap and water will also reduce diarrhea caused by *Giardia* and *Entamoeba*, as well as other parasites, bacteria, and viruses, this solution is unavailable to hundreds of millions of people who lack access to abundant clean water (41–43).

There is presently no human vaccine for *Giardia* or *Entamoeba*. While drugs to treat *Giardia* (albendazole and metronidazole) and *Entamoeba* (metronidazole) are inexpensive and efficacious, the broader public health goal is to prevent infections by whatever reasonable means are available (44). This is not to diminish the appropriate excitement about new anti-*Giardia* drugs that (i) overcome antibiotic resistance (e.g., derivatives of nitroimidazoles and benzimidazoles), (ii) have already been approved for other indications (e.g., auranofin), or (iii) target enzymes essential to parasite viability that are absent from the host (e.g., arginine deaminase) (reviewed in reference 45).

Finding drugs that inhibit cyst formation, which might break the life cycle of each parasite, faces three difficulties (46). First, trophozoites are the dividing forms that cause diarrhea (*Giardia*) or dysentery (*Entamoeba*), so inhibition of wall formation would temporarily stop shedding of infectious cysts but would not clear the primary infections. Second, the search for chitin synthase inhibitors to treat fungal infections has produced candidate compounds (e.g., nikkomycin Z), none of which is in clinical use (47). It is therefore unlikely that an inhibitor for the *Entamoeba* chitin synthase will be easily found, and the synthase that makes the GalNAc polymer in *Giardia* cyst walls has not been molecularly characterized (15). Third, because *Giardia* is a zoonotic infection, inhibitors of cyst wall formation would also need to treat animal reservoirs, which are most likely not household pets but are likely infected production animals (6–8).

**Caveats.** Only a few alcohol-based hand sanitation solutions were modeled here, so there are likely hand sanitizers with additional ingredients (e.g., detergents, organic acids, or quaternary ammonium compounds) that might be even more effective or last longer after washing. Because we were handling sets of parasites, organisms were treated with alcohol for 5 min and dried for 5 min, which is longer than it takes to use hand sanitizers. *G. duodenalis* cysts were not directly applied to the hands and washed with alcohol-based sanitizers. *E. invadens* was used to model *E. histolytica* cysts, and we did not validate alcohol treatments in an animal model (infecting reptiles with *E. invadens* cysts is beyond our capabilities).

While these hand-sanitizing solutions might reduce food contamination by infected handlers, they would not be expected to reduce waterborne infections with cysts of *Giardia* or *Entamoeba* (1, 30). Hand washing or use of hand sanitizers is uneven at best in day care centers and homes in developed countries and is likely sporadic in developing countries (2–5, 26, 28–32). The role of alcohol-based hand sanitizers in schools, day care centers, and homes in developed countries, where water is plentiful for washing hands with antimicrobial soaps, is unclear (30, 32). The dermatologic effects of alcohols (e.g., dryness) may reduce their routine use to prevent infections with *Giardia* and *Entamoeba* (48). Finally, because hand sanitizers cost more than soap and water, their use would likely need to be subsidized and targeted (e.g., to mothers or caregivers of infants).

Despite these caveats, it appears that expanded use of alcohol-based hand sanitizers, which rapidly kill cysts, has a reasonable chance of reducing transmission of *Giardia* and *Entamoeba*.

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